Guggulsterone, a Farnesoid X Receptor Antagonist, Inhibits Constitutive and Inducible STAT3 Activation through Induction of a Protein Tyrosine Phosphatase SHP-1

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Abstract

Signal transducers and activator of transcription 3 (STAT3) is a transcription factor that has been associated with survival, proliferation, chemoresistance, and angiogenesis of tumor cells. Whether the apoptotic, antiproliferative, and antimetastatic effects of guggulsterone (GS), a farnesoid X receptor antagonist, are linked to its ability to suppress STAT3 activation was investigated. We found that the *Z* but not the *E* stereoisomer of GS inhibited both constitutive and interleukin-6–induced STAT3 activation in human multiple myeloma cells. The suppression of STAT3 was mediated through the inhibition of activation of protein tyrosine kinases Janus-activated kinase 2 and c-Src. Vanadate treatment reversed the GS-induced down-regulation of STAT3, suggesting the involvement of a protein tyrosine phosphatase. Indeed, we found that GS induced the expression of both the protein and mRNA for tyrosine protein phosphatase SHP-1 that was not due to demethylation of the SHP-1 promoter previously implicated in the epigenetic silencing of SHP-1. Moreover, knockdown of SHP-1 by small interfering RNA suppressed the effect of GS on induction of SHP-1 and on the inhibition of STAT3 activation, thereby implicating SHP-1 in the action of GS. Finally, GS down-regulated the expression of STAT3-regulated antiapoptotic (*Bcl-2, Bcl-xL*, and *Mcl-1*), proliferative (*cyclin D1*), and angiogenic (*VEGF*) gene products; and this correlated with suppression of proliferation, the accumulation of cells in sub-G_1 phase of cell cycle, and induction of apoptosis. Overall, these results suggest that GS is a novel blocker of STAT3 activation and thus may have a potential in regulation of growth and metastasis of tumor cells. [Cancer Res 2008;68(11):4406–15]

Introduction

Guggulsterone (GS), derived from *Commiphora mukul* and used to treat obesity, diabetes, hyperlipidemia, atherosclerosis, and osteoarthritis (1, 2), has been recently shown to antagonize the farnesoid X receptor and decrease the expression of bile acid–activated genes (3). GS has also been shown to exhibit anticancer potential as indicated by its ability to suppress the proliferation of a wide variety of human tumor cell types, including leukemia, head and neck carcinoma, multiple myeloma, lung carcinoma, melanoma, breast carcinoma, prostate and ovarian carcinoma; induce apoptosis; and reverse chemoresistance (4–8). However, the mechanism by which GS mediates anticancer activities is not fully understood. Because signal transducer and activator of transcription (STAT) activation pathway has been closely linked with proliferation, apoptosis, and chemoresistance, it is possible that GS mediates its effects through modulation of this pathway.

STAT proteins were originally discovered as latent cytoplasmic transcription factors a decade ago (9). There are seven known mammalian STAT proteins, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6, which are involved in cell proliferation, differentiation, and apoptosis (10, 11). One STAT family member, STAT3, is often constitutively active in many human cancer cells, including multiple myeloma, leukemia, lymphoma, and solid tumors (12, 13). STAT3 can also be activated by certain interleukins (e.g., IL-6) and growth factors (e.g., EGF). Upon activation, STAT3 undergoes phosphorylation-induced homodimerization, leading to nuclear translocation, DNA binding, and subsequent gene transcription. The phosphorylation is mediated through the activation of non–receptor protein tyrosine kinases called Janus-like kinase (JAK). JAK1, JAK2, JAK3, and TYK2 have been implicated in the activation of STAT3 (14, 15). In addition, the role of c-Src kinase has been shown in STAT3 phosphorylation (16).

The major phosphorylation sites in STAT3 include tyrosine and serine residues at positions 705 and 727, respectively, located in the transactivation domain. STAT3 participates in oncogenesis through up-regulation of genes encoding apoptosis inhibitors (*Bcl-xL, Mcl-1*, and *survivin*), cell cycle regulators (*cyclin D1* and *c-Myc*), and inducers of angiogenesis [vascular endothelial growth factor (VEGF); ref. 13]. Bcl-xL is an antiapoptotic protein within the Bcl-2 family that inhibits apoptosis by binding proapoptotic proteins and preventing cytochrome *c* release (17, 18). High levels of Bcl-xL expression were associated with advanced disease and poor prognosis in several tumor systems (19). Mcl-1 also represents a survival factor for human cancer cells (20). Suppression of the expression of Bcl-xL and Mcl-1 proved to be useful in cancer therapy by inducing cell apoptosis. STAT3 is activated in many human cancers, including 82% of prostate cancers (21), 70% of breast cancers (22), >82% of squamous cell carcinoma of the head and neck (SCCHN; ref. 23), and 71% of nasopharyngeal carcinoma (24). In SCCHN, STAT3 has been found to alter the cell cycle, prevent apoptosis, and mediate the proliferation and survival of tumor cells (25). Thus, agents that suppress STAT3 activation have implication for prevention and treatment of cancer (26).

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Because of the critical role of STAT3 activation in tumor cell survival, proliferation, and chemoresistance, we hypothesized that GS mediates its effects through the suppression of STAT3 pathway. The results indicate that GS indeed suppressed both constitutive and inducible STAT3 activation and down-regulated expression of cell survival, proliferative, and angiogenic gene products, leading to suppression of proliferation and induction of apoptosis.

Materials and Methods

Reagents. (*Z*)-GS and (*E*)-GS, obtained from Steraloids, Inc., were dissolved in DMSO as a 10 mmol/L stock solution and stored at −20°C. Further dilution was done in cell culture medium. RPMI 1640, fetal bovine serum (FBS), 0.4% trypan blue vital stain, and antibiotic-antimycotic mixture were obtained from Life Technologies. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Tris, glycine, NaCl, SDS, and bovine serum albumin were purchased from Sigma-Aldrich. Rabbit polyclonal antibodies to STAT3 and mouse monoclonal antibodies against phospho-STAT3 (Tyr^{705}) and Bcl-2, Bcl-xL, Mcl-1, SHP-1, procaspase-3, and poly(ADP)ribose polymerase (PARP) were obtained from Santa Cruz Biotechnology. Goat anti-rabbit–horseradish peroxidase (HRP) conjugate was purchased from Bio-Rad. Antivascular endothelial growth factor (VEGF) was purchased from NeoMarkers. Antibodies to phosphospecific Src (Tyr^{416}), Src, and JAK2 were purchased from Cell Signaling Technology. GST-JAK2 was kindly provided by Dr. Z.J. Zhao (University of Oklahoma Health Sciences Center, Tulsa, OK). Goat anti-mouse HRP was purchased from Transduction Laboratories. The siRNA for SHP-1 and the scrambled control were obtained from Ambion.

Cell lines. Human multiple myeloma cell lines U266, MM.1S (melphalan-sensitive), A293 (human embryonic kidney), and head and neck squamous cell carcinoma SCC4 were obtained from the American Type Culture Collection. Cell line U266 (ATCC TIB-196) is a plasmacytoma of B-cell origin and is known to produce monoclonal antibodies and IL-6. The MM.1S cell line, established from the peripheral blood cells of a patient with IgA myeloma, secretes λL chain, is negative for the presence of the EBV genome, and expresses leukocyte antigen DR, plasma cell Ag-1, and T9 and T10 antigens (27). U266 and MM.1S cells were cultured in RPMI 1640 containing 10% FBS. A293 cells were cultured in DMEM supplemented with 10% FBS. SCC4 cells were cultured in DMEM containing 10% FBS, nonessential amino acids, pyruvate, glutamine, and vitamins. All media were also supplemented with 100 units/mL of penicillin and 100 μg/mL of streptomycin.

Electrophoretic mobility shift assay for STAT3-DNA binding. STAT3-DNA binding was analyzed by electrophoretic mobility shift assay (EMSA) using a ^{32}P-labeled high-affinity sis-inducible element (hSIE) probe (5′-CT-TCATTTCCCGTAAATCCCTAAAGCT-3′ and 5′-AGCTTTAGGGATTTACGG-GAAATGA-3′) as previously described (28). Briefly, nuclear extracts were prepared from GS-treated cells and incubated with the hSIE probe. The DNA-protein complex formed was separated from free oligonucleotide on 5% native polyacrylamide gels. The dried gels were visualized, and the radioactive bands were quantitated with a Storm 820 and Imagequant software (Amersham).

Western blotting. For detection of STAT proteins, GS-treated whole-cell extracts were lysed in lysis buffer [20 mmol/L Tris (pH 7.4), 250 mmol/L NaCl, 2 mmol/L EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/mL aprotinin, 0.005 mg/mL leupeptin, 0.4 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 4 mmol/L $NaVO_4$]. Lysates were then spun at 14,000 rpm for 10 min to remove insoluble material and resolved on a 10% SDS-PAGE. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with anti-STAT antibodies (1:1,000) overnight at 4°C. The blot was washed, exposed to HRP-conjugated secondary antibodies for 2 h, and finally examined by enhanced chemiluminescence (ECL; Amersham).

To detect STAT3-regulated proteins and caspase-3, U266 cells (1×10^6/mL) were treated with GS for the indicated times. The cells were then washed and extracted by incubation for 30 min on ice in 0.1 mL buffer containing 20 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 250 mmol/L NaCl, 0.1% NP40, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mmol/L PMSF, 0.5 μg/mL benzamidine, 1 mmol/L DTT, and 1 mmol/L sodium vanadate. The lysate was centrifuged and the supernatant was collected. Whole-cell extract protein (30 μg) was resolved on 10% SDS-PAGE; electrotransferred onto a nitrocellulose membrane; blotted with antibodies against Bcl-2, Bcl-xl, cyclin D1, VEGF, Mcl-1, or caspase-3; and then examined by ECL (Amersham).

STAT3 luciferase reporter assay. A293 cells were plated in six-well plates with 5×10^5 per well in DMEM containing 10% FBS. The STAT3-responsive elements linked to a luciferase reporter gene was transfected with wild-type or dominant-negative STAT3-Y705F (STAT3F).

Transfections were done according to the manufacturer's protocols using Fugene-6 (Roche). At 24 h posttransfection, cells were pretreated with GS for 4 h and then induced by IL-6 for additional 24 h before being washed and lysed in luciferase lysis buffer (Promega). Luciferase activity was measured with a luminometer by using a luciferase assay kit (Promega) and was normalized to β-galactosidase activity. All luciferase experiments were done in triplicate and repeated three or more times. The data show the mean and the SD of the mean of the experiments.

JAK2 kinase assay. Cells were lysed for 30 min on ice in whole-cell lysis buffer [20 mmol/L HEPES (pH 7.9), 50 mmol/L NaCl, 1% NP40, 2 mmol/L EDTA, 0.5 mmol/L EGTA, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 0.5 mmol/L PMSF, and 2 mmol/L sodium orthovanadate]. Lysate containing 900 μg of proteins in lysis buffer was incubated with 1 μg/mL concentration of JAK2 antibody overnight. Immunocomplex was precipitated using protein A/G agarose beads for 2 h at 4°C. After 2 h, the beads were washed with lysis buffer and then resuspended in a kinase assay mixture containing 50 mmol/L HEPES (pH 7.4), 20 mmol/L $MgCl_2$, 2 mmol/L DTT, 20 μCi [γ-^{32}P]ATP, 10 μmol/L unlabeled ATP, and 2 μg of substrate GST-JAKs. After incubation at 30°C for 30 min, the reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized with a Storm820. To determine the total amounts of JAK2 in each sample, 30 μg of whole-cell proteins were resolved on 10% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with anti-JAK2 antibody.

MTT assay. The antiproliferative effect of GS against multiple myeloma (MM) cell lines was determined by the MTT dye uptake method as described earlier (29).

Transfection with SHP-1 siRNA. SCC4 cells were plated in each well of six-well plates and allowed to adhere for 24 h. On the day of transfection, 12 μL Hiperfect transfection reagent (Qiagen) were added to 50 nmol/L SHP-1 siRNA in a final volume of 100 μL culture medium. After 48 h of transfection, cells were treated with GS for 4 h and whole-cell extracts were prepared for SHP-1, STAT3, and phospho-STAT3 analysis by Western blot.

Immunoblot analysis of PARP degradation. GS-induced apoptosis was examined by proteolytic cleavage of PARP. Briefly, cells (1×10^6/mL) were treated with GS for the indicated times at 37°C. The cells were then washed and extracted by incubation for 30 min on ice in 0.1 mL buffer containing 20 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 250 mmol/L NaCl, 0.1% NP40, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mmol/L PMSF, 0.5 μg/mL benzamidine, 1 mmol/L DTT, and 1 mmol/L sodium vanadate. The lysate was centrifuged and the supernatant was collected. Cell extract protein (30 μg) was resolved on 7.5% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, blotted with anti-PARP antibody, and then detected by ECL (Amersham).

RNA analysis and reverse transcription-PCR. U266 cells were left untreated or treated with GS for various times, washed, and suspended in Trizol reagent. Total RNA was extracted according to the manufacturer's instructions (Invitrogen, Life Technologies). One microgram of total RNA was converted to cDNA by Superscript reverse transcriptase and then amplified by Platinum Taq polymerase using Superscript One Step reverse transcription-PCR (RT-PCR) kit (Invitrogen). The relative expression of SHP-1, cyclin D1, and Bcl-2 was analyzed using quantitative RT-PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal

control. The RT-PCR reaction mixture contained 12.5 μL of 2× reaction buffer, 10 μL each of RNA, 0.5 μL each of forward and reverse primers, and 0.5 μL of RT-Platinum Taq in a final volume of 24 μL. The reaction was performed at 50°C for 30 min, 94°C for 2 min, 94°C for 30 cycles of 15 s each, 55°C for 30 s, and 72°C for 1 min with extension at 72°C for 10 min. PCR products were run on 2% agarose gel and then stained with ethidium bromide. Stained bands were visualized under UV light and photographed.

Thymidine incorporation assay. The cell proliferative effects were also monitored by the thymidine incorporation method. Briefly, 1,000 cells in 100 μL of medium were cultured in triplicate in 96-well plates with various concentrations of GS for 2 or 4 d at 37°C. Six hours before the completion of the experiment, cells were pulsed with 0.5 μCi (0.0185 mBq) of [^{3}H]thymidine, and the uptake of [^{3}H]thymidine was monitored with a Matrix-9600 β-counter (Packard Instrument Co.).

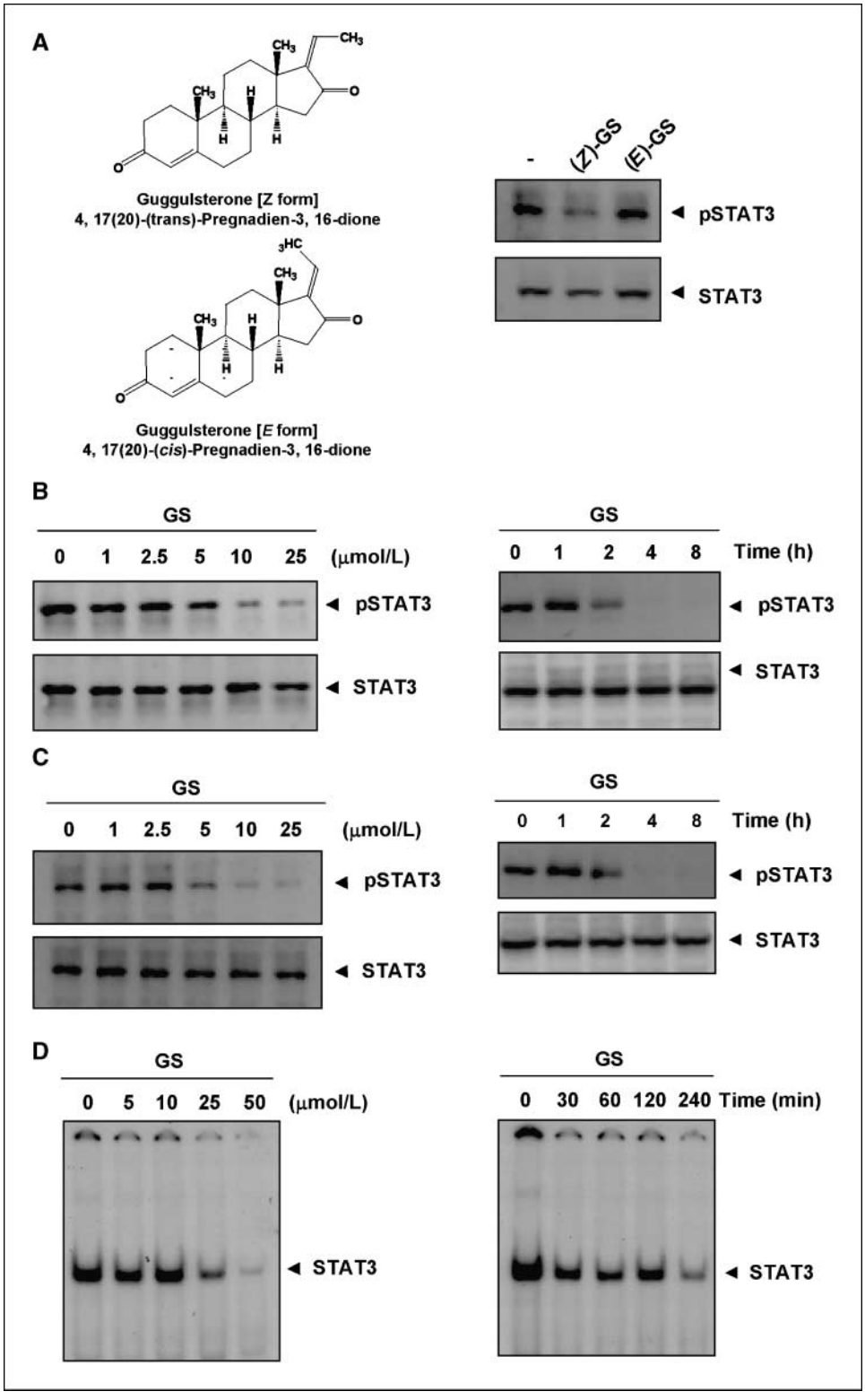

Figure 1. *A,* the structures of (*Z*)- and (*E*)-GS (*left*) and only (*Z*)-GS suppresses phospho-STAT3 levels in MM cells (*right*). U266 cells (1×10^6/mL) were treated with the 25 μmol/L (*Z*)- or (*E*)-GS for 4 h, after which whole-cell extracts were prepared and 30 μg of protein were resolved on 10% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3. *B,* U266 cells (1×10^6/mL) were treated with the indicated concentrations of GS for 4 h, after which whole-cell extracts were prepared, and 30 μg of protein were resolved on 10% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading (*left*) and GS suppresses phospho-STAT3 levels in a time-dependent manner. U266 cells (1×10^6/mL) were treated with the 25 μmol/L GS for the indicated times, after which Western blotting was performed as described previously. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading (*right*). *C,* GS suppresses phospho-STAT3 levels in a dose-dependent manner in human head and neck squamous cell carcinoma (*left*) and GS suppresses phospho-STAT3 levels in a time-dependent manner in human head and neck squamous cell carcinoma (*right*). SCC4 cells (1×10^6/mL) were treated with the 25 μmol/L GS for the indicated times, after which Western blotting was performed as described previously. *D,* GS inhibits constitutively active STAT3 in U266 cells. U266 cells (2×10^6/mL) were treated with the indicated concentrations of GS for 4 h and analyzed for nuclear STAT3 levels by EMSA (*left*) and U266 cells (2×10^6/mL) were treated with 25 μmol/L GS for the indicated durations and analyzed for nuclear STAT3 levels by EMSA. GS suppresses phospho-STAT3 levels in a time-dependent manner (*right*).

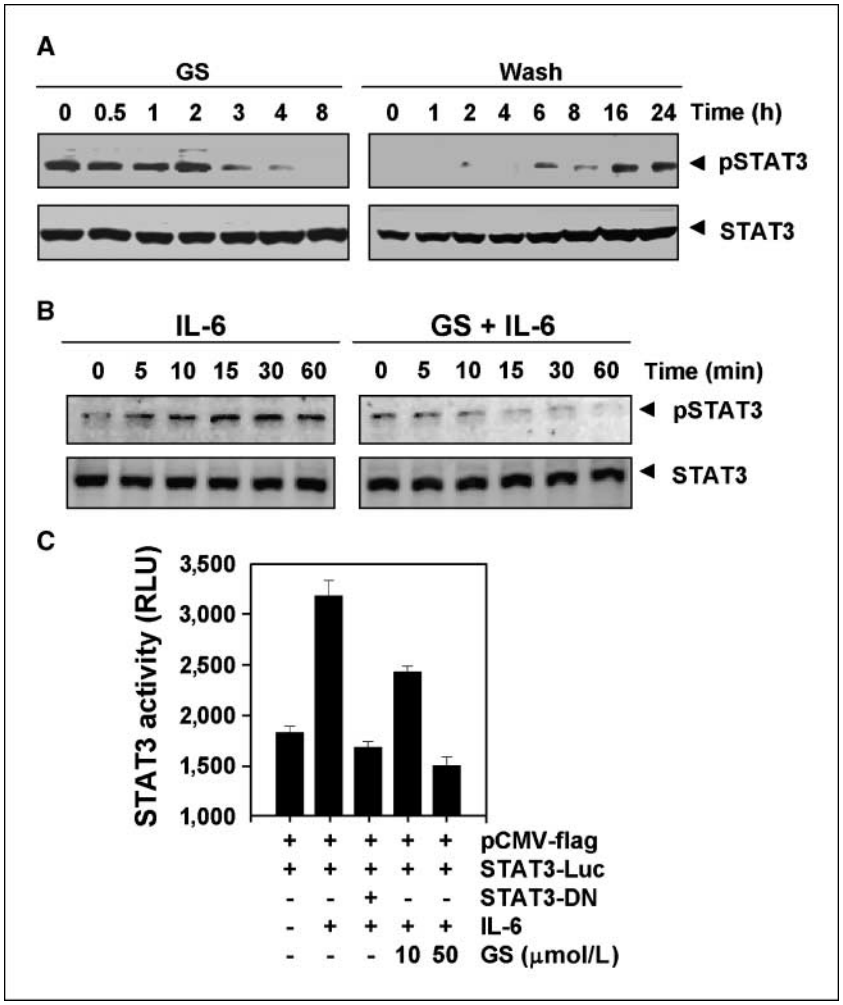

Figure 2. *A,* GS-induced inhibition of STAT3 phosphorylation is reversible. U266 cells (1×10^6) were treated with 25 μmol/L GS for the indicated durations (*left*) or treated for 1 h and washed with PBS twice to remove GS before resuspension in fresh medium (*right*). Cells were removed at indicated times and lysed to prepare the whole-cell extract. Thirty micrograms of whole-cell extracts were resolved on 10% SDS-PAGE, electrotransferred to a nitrocellulose membrane, probed for the phosphorylated STAT3 (pSTAT3), and stripped and reprobed for STAT3 antibodies. *B,* GS down-regulates IL-6–induced phospho-STAT3. MM1.S cells (2×10^6/mL) were treated with IL-6 (10 ng/mL) for indicated times, whole-cell extracts were prepared, and phosphorylated STAT3 was detected by Western blot as described in Materials and Methods. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. *C,* GS suppresses IL-6–induced STAT3 activity. A293 cells (5×10^5/mL) were transfected with STAT3-luciferase (STAT3-Luc) plasmid, incubated for 24 h, and treated with 10 and 50 μmol/L GS for 4 h and then stimulated with IL-6 (10 ng/mL) for 24 h. Whole-cell extracts were then prepared and analyzed for luciferase activity. Cells were cotransfected with β-gal and the data were normalized with β-galactosidase assay (data not shown). The results shown are representative of three independent experiments.

DNA extraction, bisulfite modification, and methylation-specific PCR assay for SHP1. U266 and SCC4 were seeded at 5×10^5 cells/six-well culture dish in appropriate growth medium. Following 24 h of growth, cells were treated for various time points with 25 μmol/L GS and 3 μmol/L of 5-Aza-2′-deoxycytidine (5-AzaDC) for 4 h. The cells were washed with PBS and genomic DNA was extracted using QIAamp DNA extraction kit (Qiagen, Inc.) according to the manufacturer's instructions. Genomic DNA was subsequently bisulfite modified for methylation analysis using EZ-DNA Methylation Gold kit (Zymo Research). MSP was performed on bisulfite-modified DNA using primers specific for either the methylated or the modified unmethylated *SHP-1* promoter P2-region. Primer sequences for the unmethylated reaction were 5′-GTGAATGTTATTATAGTATAGTGTTTGG-3′ (sense) and 5′-TCACACATACAAACCCAAACAAT-3′ (antisense), and primer sequences for the methylated reaction were 5′-GAACGTTATTATAGTA-TAGCGTTC-3′ (sense) and 5′-TCACGCATACGAACCCAAACG-3′ (antisense) as described previously (30). Step-down PCR reactions were performed in a 25 mL reaction volume containing 2× HotStar PCR buffer (Qiagen), 10 pmol of each primer, and 50 ng bisulfite-modified DNA. Reactions were hot-started at 95°C for 10 min. This was followed by 35 cycles of amplification at 95°C for 45 s, 60°C for 30 s, and 72°C for 30 s, followed by a 10-min extension at 72°C in a PTC 200 DNA Engine Thermocyler (MJ Research, Inc.). The amplification products were separated on a 3% agarose gel and visualized by ethidium bromide staining and UV transillumination.

Results

The present study was undertaken to determine whether GS affects STAT3 activation and if so through what mechanism. Both constitutive and IL-6–induced STAT3 activation was examined. Whether GS affects STAT3-regulated gene products, cellular proliferation, survival, and apoptosis was also investigated. The structures of the two stereoisomers of GS are shown in Fig. 1*A* (*left*). The dose (up to 25 μmol/L) and duration (0–8 h) of GS used for modulating STAT3 activation had no effect on cell viability (data not shown).

Inhibition of constitutive STAT3 phosphorylation of GS is stereoisomer specific. Two distinct stereoisomers of GS, (*E*) and (*Z*), have been identified. Whether both stereoisomers can equally suppress constitutive STAT3 phosphorylation was examined. We found that only (*Z*)-GS inhibited constitutive STAT3 activation; (*E*)-GS had no effect (Fig. 1*A, right*).

GS inhibits constitutive STAT3 phosphorylation. Human MM cells are known to express constitutively active STAT3 (12). Whether GS can modulate the constitutive STAT3 activation in these MM cells was investigated. As shown in Fig. 1*B* (*left*), GS inhibited the constitutive activation of STAT3 in a dose-dependent manner, with maximum inhibition occurring at 10 to 25 μmol/L. GS had no effect on the expression of STAT3 protein (Fig. 1*B, left, bottom*).

We also determined the incubation time with GS required for suppression of STAT3 activation in U266 cells. As shown in Fig. 1*B* (*right*), the inhibition was time dependent, with maximum inhibition occurring at around 4 h, again with no effect on the expression of STAT3 protein (Fig. 1*B, right, bottom*).

Inhibition of STAT3 phosphorylation by GS is not cell type specific. We established whether GS can modulate constitutive STAT3 activation in cells other than human MM cells. Because SCC4 have been shown to express constitutive STAT3 activation (31), we determined whether GS could inhibit this activation in these cells. In fact, it inhibited STAT3 activation in a dose-dependent manner, with maximum inhibition occurring at 10 to 25 μmol/L (Fig. 1*C, left, top*) and had no effect on the expression of STAT3 protein (Fig. 1*C, left, bottom*). As shown in Fig. 1*C* (*right*), the inhibition was time dependent, with maximum inhibition occurring at around 4 h.

GS inhibits binding of STAT3 to the DNA. Because tyrosine phosphorylation causes dimerization of STATs and their translocation to the nucleus, where they bind to DNA and regulate gene transcription (28), we determined whether GS suppresses the DNA-binding activity of STAT3. EMSA analysis of nuclear extracts prepared from U266 cells showed that GS decreased STAT3 DNA-binding activity in a dose-dependent (Fig. 1*D, left*) and time-dependent manner (Fig. 1*D, right*). These results show that GS abrogates the DNA-binding ability of STAT3.

GS-induced inhibition of STAT3 phosphorylation is reversible. We further examined whether GS-induced inhibition of STAT3 phosphorylation is reversible. Cells were first treated for various intervals with GS and then washed twice with PBS to remove the drug. The cells were then cultured in fresh medium for various durations, and the level of phosphorylated STAT3 were measured. GS induced the suppression of STAT3 phosphorylation (Fig. 2*A, left*), but after the removal of GS, phosphorylated STAT3 gradually increased (Fig. 2*A, right*). The reversal was complete by 16 h and did not involve changes in STAT3 protein levels (Fig. 2*A, bottom*).

GS also inhibits IL-6–induced STAT3 phosphorylation. Because IL-6 is a growth factor for MM cells and induces STAT3 phosphorylation (32), we determined whether GS could inhibit IL-6–induced STAT3 phosphorylation. MM.1S cells, which lack constitutively active STAT3, were treated with IL-6 for different times and then examined for phosphorylated STAT3. IL-6 induced phosphorylation of STAT3 as early as 10 min, but phosphorylation began to decline at 60 min. In MM.1S cells pretreated with GS for 4 h, IL-6–induced STAT3 phosphorylation was suppressed (Fig. 2*B*).

GS suppresses IL-6–induced STAT3-dependent reporter gene expression. Our results up to this point showed that GS inhibited the phosphorylation, nuclear translocation, and DNA-binding activity of STAT3. We next determined whether GS affects STAT3-dependent gene transcription. When cells transiently transfected with the pSTAT3-Luc construct were stimulated with IL-6, STAT3-mediated luciferase gene expression significantly increased. Dominant-negative STAT3 blocked this increase, indicating specificity. When the cells were pretreated with GS, IL-6–induced STAT3 activity was inhibited in a dose-dependent manner (Fig. 2*C*).

GS suppresses constitutive activation of JAK2. Because STAT3 is activated by soluble tyrosine kinases of the Janus family (JAK; ref. 14) and JAK2 is one of the main kinases involved, we measured the effects of GS on JAK2 activation. GS-treated whole-cell lysates were immunoprecipitated with anti-JAK2 antibodies and then subjected to an immunocomplex kinase assay using GST-JAK2 as a substrate. We found that GS suppressed the constitutive phosphorylation of JAK2 in a time-dependent manner (Fig. 3*A*). The levels of total JAK2 remained unchanged under the same conditions (Fig. 3*A, bottom*).

GS suppresses constitutive activation of c-Src. Because STAT3 is also activated by soluble tyrosine kinases of the Src kinase families (16), we determined the effect of GS on constitutive activation of c-Src kinase in U266 cells. We found that GS suppressed the constitutive phosphorylation of c-Src kinase (Fig. 3*B*). The levels of total c-Src kinase remained unchanged under the same conditions (Fig. 3*B, bottom*).

Tyrosine phosphatases are involved in GS-induced inhibition of STAT3 activation. Because protein tyrosine phosphatases have been implicated in STAT3 activation (33), we determined whether GS-induced inhibition of STAT3 tyrosine phosphorylation could be due to activation of a protein tyrosine phosphatase (PTPase). Treatment of U266 cells with the broad-acting tyrosine phosphatase inhibitor sodium pervanadate reversed the GS-induced

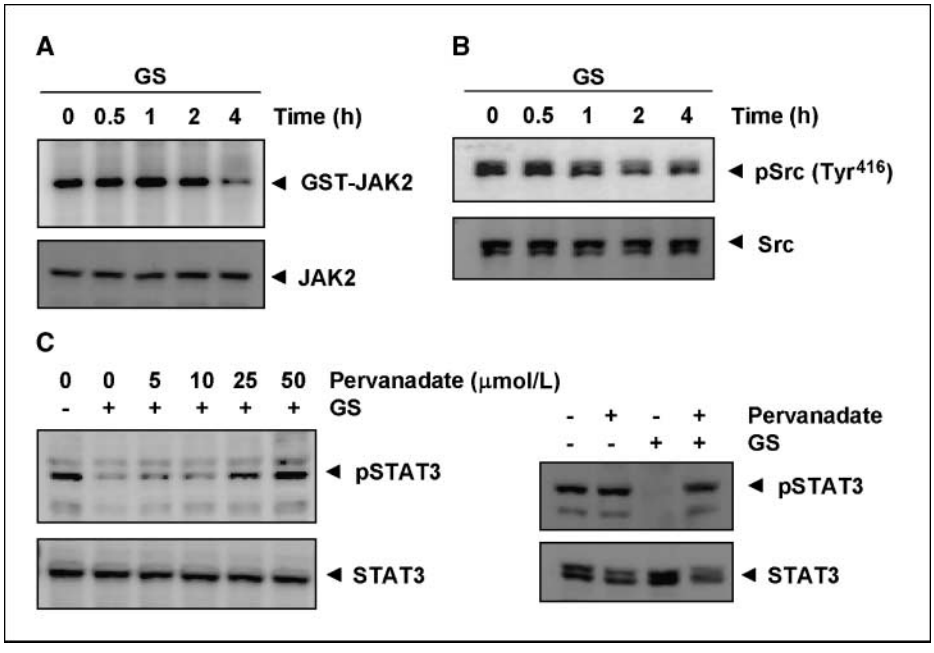

Figure 3. *A,* GS suppresses the activation of JAK2 in a time-dependent manner. U266 cells (4×10^6/mL) were treated with 25 μmol/L GS for the indicated time intervals. Whole-cell extracts were immunoprecipitated with antibody against JAK2 and analyzed by an immunocomplex kinase assay. The same samples were analyzed for JAK2 protein. *B,* GS suppresses phospho-Src levels in a time-dependent manner. U266 cells (2×10^6/mL) were treated with 25 μmol/L GS, after which whole-cell extracts were prepared and 30 μg aliquots of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed for phospho-Src antibody. The same blots were stripped and reprobed with Src antibody to verify equal protein loading. *C,* pervanadate reverses the phospho-STAT3 inhibitory effect of GS. U266 cells (2×10^6/mL) were treated with the indicated concentration of pervanadate and 25 μmol/L GS for 4 h, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3 and STAT3 (*left*). Pervanadate affects the phosphorylation level of STAT3 in the presence or absence of GS (*right*).

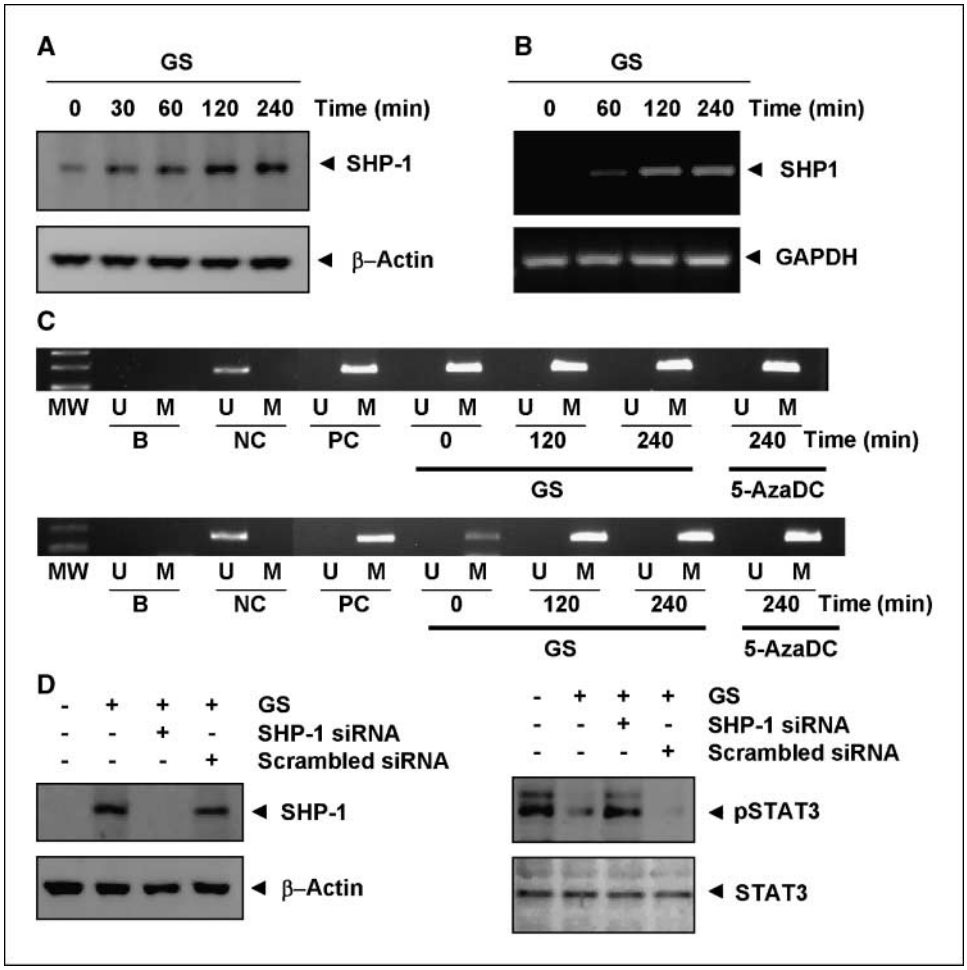

Figure 4. *A,* GS induces the expression of SHP-1 protein in U266 cells. U266 cells (2×10^6/mL) were treated with 25 μmol/L GS for indicated time intervals, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed for SHP-1 antibody. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. *B,* GS induces SHP-1 gene expression. U266 cells (4×10^6/mL) were treated 25 μmol/L (*Z*)-GS for the indicated time intervals, and total RNA was extracted and examined for expression of *SHP-1* by RT-PCR. GAPDH was used as an internal control to show equal RNA loading. *C,* GS has no effect on the methylation of SHP-1 promoter. MSP for *SHP1* promoter region in GS-treated U266 (*top*) and SCC4 (*bottom*) cell lines. *B,* water blank; *NC,* negative control DNA from the peripheral blood of a healthy volunteer; *PC,* positive control human genomic DNA artificially methylated with *SssI* methyltransferase. As indicated, both U266 and SCC4 cell lines are methylated for *SHP1*. GS treatment at various time points had no effect on reversal of *SHP1* promoter methylation. Similarly, demethylating treatment with 5-AzaDC also did not inhibit *SHP1* after 4 h. *U,* unmethylated; *M,* methylated. *D,* effect of SHP-1 knockdown on GS-induced expression of SHP-1. SCC4 cells (1×10^5/mL) were transfected with either scrambled or SHP-1-specific siRNA (50 nmol/L). After 48 h, cells were treated with 25 μmol/L GS for 4 h and whole-cell extracts were subjected to Western blot analysis for SHP-1. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading (*left*) and transfection with SHP-1 siRNA reverses GS-induced suppression of STAT3 activation. The same whole-cell extracts were subjected to phospho-STAT3 and STAT3 (*right*).

inhibition of STAT3 activation (Fig. 3*C, left*). This suggests that tyrosine phosphatases are involved in GS-induced inhibition of STAT3 activation.

Whether constitutive phosphorylation of STAT3 is modulated by pervanadate was also examined. We found that sodium pervanadate did not affect the basal level of STAT3 phosphorylation but reversed the GS-induced inhibition of STAT3 phosphorylation (Fig. 3*C, right*).

GS induces the expression of SHP-1. SHP-1, a nontransmembrane protein tyrosine phosphatase expressed in most cells (34), is important in the negative regulation of JAK/STAT signaling and is often silenced by methylation in leukemias and lymphomas (30). We therefore examined whether GS can modulate expression of SHP-1 in U266 cells. As shown in Fig. 4*A*, GS induced the expression of SHP-1 protein in a time-dependent manner, with maximum expression occurring at 120 to 240 minutes.

We also examined the effect of GS on the transcription of SHP-1. For this, cells were incubated with GS at different intervals and total RNA was examined for SHP-1 mRNA expression by RT-PCR analysis. As shown in Fig. 4*B*, GS induced the expression of SHP-1 mRNA.

GS has no effect on the demethylation of SHP-1 promoter. DNA methylation of the SHP-1 promoter has been shown to be responsible for epigenetic silencing of SHP-1 in leukemia and lymphoma cells (30, 35). Whether GS induces SHP-1 through demethylation of the SHP-1 promoter was investigated in U266 and in SCC4 cells. To determine this, cells were treated for various time points with GS or 5-AzaDC (a known inhibitor of demethylation), and analyzed for demethylation as described in Materials and Methods. The results indicated that these two cancer cell lines exhibited constitutively methylated *SHP1* promoter and GS had no effect on the methylation under the conditions (2–4 hours) the

mRNA for SHP-1 was induced (Fig. 4*C*), thus suggesting that effect of GS on SHP-1 mRNA expression is not due to demethylation. 5-Aza-DC also had no effect on *SHP-1* methylation at 4 hours, as Chim and colleagues (36) showed that treatment of cells with 5-Aza-DC for 5 days is needed to modulate promoter methylation.

SHP-1 siRNA down-regulate the expression of SHP-1 and reverses the inhibition of STAT3 activation by GS. We determined whether the suppression of SHP-1 expression by siRNA would abrogate the inhibitory effect of GS on STAT3 activation. Western blotting showed that GS-induced SHP-1 expression was effectively abolished in the cells treated with SHP-1 siRNA; treatment with scrambled siRNA had no effect (Fig. 4*D, left*). We also found that GS failed to suppress STAT-3 activation in cells treated with SHP-1 siRNA (Fig. 4*D, right*). These siRNA results corroborate our earlier evidence of the critical role of SHP-1 in suppression of STAT-3 phosphorylation by GS.

GS suppresses the expression of proliferative gene product. Cyclin D1, which is required for cell proliferation and for transition from G_1 to S phase of the cell cycle, is regulated by STAT3 (37). We showed that GS treatment suppressed the expression of cyclin D1 in a time-dependent manner (Fig. 5*A*).

GS down-regulates the expression of antiapoptotic gene products. STAT3 activation also regulates the expression of various gene products involved in cell survival, including Bcl-2, Bcl-xL, and Mcl-1 (37, 38). As was the case for cyclin D1, we found that GS treatment down-regulated expression of these antiapoptotic proteins in a time-dependent manner, with maximum suppression observed at around 16 to 24 h (Fig. 5*B*).

GS down-regulates the expression of angiogenic gene product. VEGF, a major mediator of angiogenesis, is also regulated by STAT3 activation (39, 40). GS treatment down-regulated expression of VEGF in a time-dependent manner (Fig. 5*C*).

The suppression of gene products by GS occurs at the transcriptional level. Whether GS affects the transcription of *cyclin D1* and *Bcl-2* was examined. The mRNA of both the genes was constitutively expressed, and GS treatment suppressed the expression in a time-dependent manner (Fig. 5*D*). These results suggest that GS modulates the expression of genes at the transcriptional level.

GS inhibits proliferation of MM cells. Because GS suppressed the activation of STAT3 and STAT3-regulated gene products, whether it modulates proliferation of cells was examined. Results in Fig. 6*A* (*left* and *right*) show that GS suppressed the increase in proliferation of U266 cells in a time- and dose-dependent manner.

GS causes the accumulation of the cells in the sub-G_1 phase of the cell cycle. Because D-type cyclins are required for the progression of cells from the G_1 phase of the cell cycle to S phase (41) and rapid decline in levels of cyclin D1 was observed in GS-treated cells, we determined the effect of GS on cell cycle phase distribution. After treatment for 36 hours, GS induced accumulation of 17% of cell population in the sub-G_1 phase, which is indicative of apoptosis (Fig. 6*B*).

GS activates caspase-3 and causes PARP cleavage. Whether suppression of constitutively active STAT3 in U266 cells by GS leads to apoptosis was also investigated. Cells were treated with GS for different times and then examined for caspase activation by Western blot using specific antibodies. We found a time-dependent activation of caspase-3 by GS (Fig. 6*C*). Activation of downstream caspases led to the cleavage of a 116 kDa PARP protein into an 87 kDa fragment (Fig. 6*D, left*). No PARP cleavage occurred in control cells at 24 or 48 h (Fig. 6*D, right*). These results clearly suggest that GS induces caspase-3–dependent apoptosis in U266 cells.

Discussion

Although GS has been shown to suppress the proliferation of a wide variety of cell types and induce apoptosis, the mechanism is not fully understood. The goal of this study was to investigate the

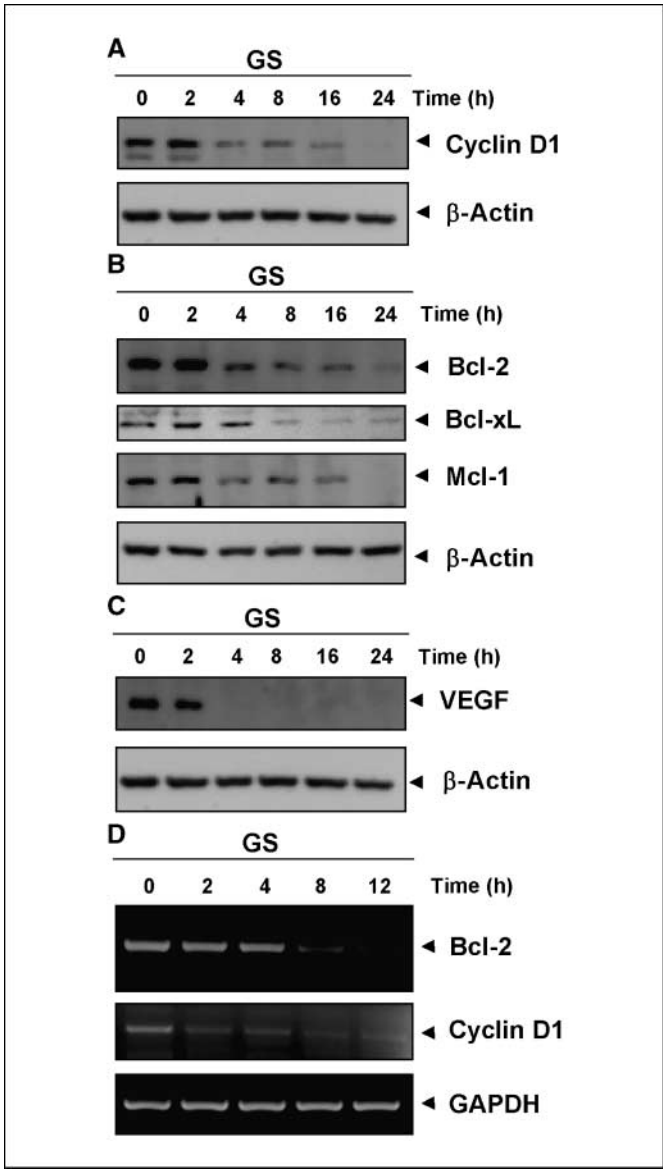

Figure 5. *A,* GS suppresses STAT3-regulated proliferative gene product. U266 cells (2×10^6/mL) were treated with 25 μmol/L GS for indicated time intervals, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS-PAGE; the membrane was sliced according to molecular weight and probed against cyclin D1 antibody. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. *B,* GS suppresses the expression of Bcl-2, Bcl-xL, and Mcl-1 protein. U266 cells (2×10^6/mL) were treated with 25 μmol/L GS for the indicated time intervals, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS-PAGE and probed against Bcl-2, Bcl-xL, and Mcl-1 antibodies. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. *C,* GS suppresses STAT3-regulated angiogenic gene product. U266 cells (2×10^6/mL) were treated with 25 μmol/L GS for the indicated time intervals, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS-PAGE; the membrane was sliced according to molecular weight and probed against the VEGF antibody. *D,* GS inhibits gene expression. U266 cells (4×10^6/mL) were treated with GS (25 μmol/L) for the indicated time points, and total RNA was extracted and examined for expression of *Bcl-2* and *cyclin D1* by RT-PCR. GAPDH was used as an internal control to show equal RNA loading.

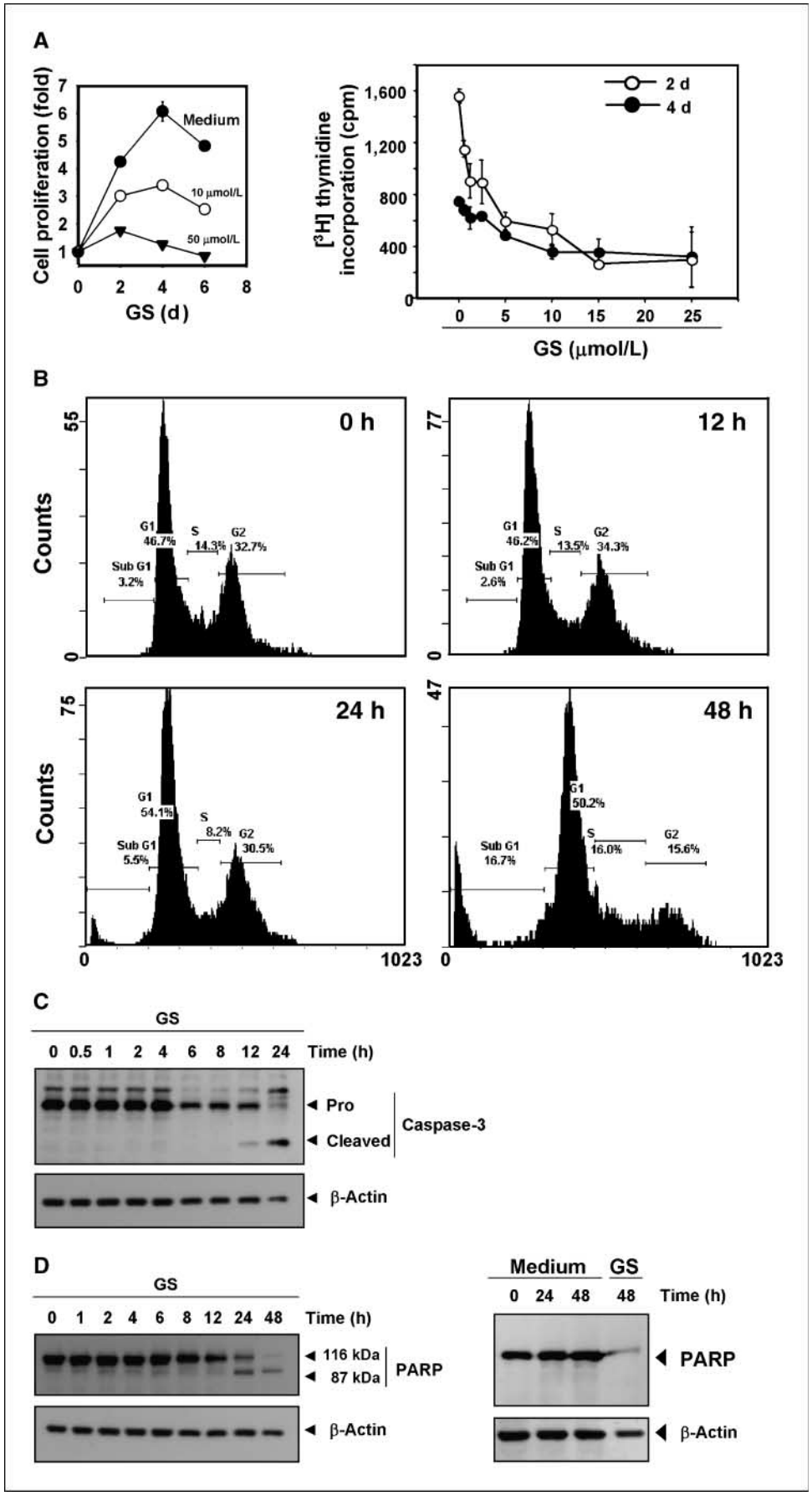

Figure 6. *A,* GS suppresses cell viability in multiple myeloma. U266 cells were plated in triplicate, treated with 10 and 50 μmol/L GS, and then subjected to MTT assay on days 0 to 6 to analyze proliferation of cells (*left*) and GS suppresses cell proliferation in multiple myeloma. U266 cells were plated in triplicate and treated with the indicated concentrations of GS for 2 or 4 d (*right*). Cell proliferation assays were performed as described in Materials and Methods. Results are shown as the cpm mean ± SD [^{3}H]thymidine incorporation from triplicate cultures. *B,* GS causes significant accumulation of cells in the sub-G_1 phase. U266 cells (2×10^6/mL) were synchronized by incubation overnight in the absence of serum and then treated with 25 μmol/L GS for the indicated times, after which the cells were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. *C,* GS induces caspase-3 activation. U266 cells were treated with 25 μmol/L GS for the indicated times, and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blotting against caspase-3 antibody. The same blot were stripped and reprobed with β-actin antibody to show equal protein loading. *D,* GS causes PARP cleavage. U266 cells were treated with 25 μmol/L GS for the indicated times, and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot against PARP antibody (*left*). Untreated cells fail to induce the PARP cleavage at 24 or 48 h (*right*). The same blots were stripped and reprobed with β-actin antibody to show equal protein loading. The results shown are representative of three independent experiments.

effect of GS on the STAT3 signaling, gene products, and cellular responses. We found that this agent suppressed both constitutive and IL-6–induced STAT3 activation. This effect was not cell type specific as STAT3 activation in both human MM and head and neck squamous carcinoma cells was inhibited. Interestingly, however, the effect of GS was stereoisomer specific. Inhibition of STAT3 activation by GS involved the inhibition of c-Src and JAK2 activation. We further found that GS stimulated the expression of protein tyrosine phosphatase SHP-1, and this was essential for the down-regulation of STAT3. GS also down-regulated the expression of STAT3-regulated gene products, including *cyclin D1, Bcl-2, Bcl-xL, Mcl-1*, and *VEGF*; and inhibited the proliferation of cells, accumulation of cells in G_1-G_0 phase, and apoptosis.

We found for the first time that GS could suppress both constitutive and inducible STAT3 activation in MM and head and neck squamous carcinoma cells and that these effects were specific to STAT3 activation, as GS had no effect on STAT5 phosphorylation (data not shown) We also observed that GS suppressed nuclear translocation and DNA-binding activity of STAT3. How GS affects STAT3 activation was also investigated. The effects of GS on STAT3 phosphorylation correlated with the suppression of upstream protein tyrosine kinases JAK2 and c-Src. All Src-transformed cell lines have persistently activated STAT3, and dominant-negative STAT3 blocks transformation (42, 43).

We also found evidence that the GS-induced inhibition of STAT3 activation involves a protein tyrosine phosphatase (PTP). Several PTPs have been implicated in STAT3 signaling, including SHP-1 (44), SHP-2 (45), TC-PTP (46), PTEN (47), PTP-1D (48), CD45 (49), and PTP-epsilon (50); loss of SHP-1 has been shown to enhance JAK3/STAT3 signaling in ALK-positive anaplastic large-cell lymphoma (33). Indeed, we found for the first time that GS stimulates the expression of SHP-1 protein and mRNA in U266 cells, which correlated with down-regulation of constitutive STAT3 phosphorylation in these cells. Although epigenetic silencing of SHP-1 through promoter methylation has been reported (30, 35), we found that the induction of SHP-1 by GS was not due to promoter demethylation by GS. Transfection with SHP-1 siRNA abolished the STAT3 inhibitory effect of GS, thereby further implicating this phosphatase in GS-induced down-regulation of STAT3 activation.

Our group has previously shown that GS is a potent inhibitor of nuclear factor-κB (NF-κB) activation (5, 8). We do not know whether suppression of STAT3 by GS is linked with suppression of NF-κB. The p65 subunit of NF-κB has been shown to communicate with STAT3 (51), but activation of STAT3 and NF-κB are dependent on different cytokines. Although IL-6 is a major activator of STAT3, tumor necrosis factor is a potent activator of NF-κB. Interestingly, JAK2 kinase needed for STAT3 activation has been shown to be required for erythropoietin-induced NF-κB activation (52). Thus, it is possible that the suppression of JAK2 activation is the potential link for inhibition of both NF-κB and STAT3 activation by GS.

We found that the expression of several STAT3-regulated gene products was suppressed by GS. These included proliferative (*cyclin D1*) and antiapoptotic (*Bcl-2, Bcl-xL*, and *Mcl-1*) and angiogenic (*VEGF*) gene products. Constitutively active STAT3 can contribute to oncogenesis by protecting cancer cells from apoptosis; this implies that suppression of STAT3 activation by GS could facilitate apoptosis. Constitutively active STAT3 has been implicated in the induction of resistance to apoptosis (53), possibly through the expression of Bcl-2, Bcl-xL, and cyclin D1 (54). The down-regulation of cyclin D1 expression by GS correlated with suppression in proliferation and accumulation of cells in G_1 phase of cell cycle, which is consistent with the requirement of cells for cyclin D1. Expression of Bcl-xL is regulated by STAT3, and this protein is overexpressed in MM cells (55). The down-regulation of the expression of Bcl-2 and Bcl-xL could contribute to the ability of GS to induce apoptosis in MM cells. We further observed that GS induced the down-regulation of Mcl-1 protein in U266 cells.

Overall, our results show that GS inhibits inducible and constitutive STAT3 activation through the induction of tyrosine phosphatase, which makes it a potentially effective suppressor of tumor cell survival, proliferation, and angiogenesis. Further study of GS may provide important leads for potential treatment applications of cancer and other diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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